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Expression and processing of the TMEM70 protein

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ABSTRACT

TMEM70 protein represents a novel ancillary factor of mammalian ATP synthase. We have investigated import and processing of this factor in human cells using GFP- and FLAG-tagged forms of TMEM70 and specific antibodies. TMEM70 is synthesized as a 29 kDa precursor protein that is processed to a 21 kDa mature form. Immunocytochemical detection of TMEM70 showed mitochondrial colocalization with MitoTracker Red and ATP synthase. Western blot of subcellular fractions revealed the highest signal of TMEM70 in isolated mitochondria and mitochondrial location was confirmed by mass spectrometry analysis. Based on analysis of submitochondrial fractions, TMEM70 appears to be located in the inner mitochondrial membrane, in accordance with predicated transmembrane regions in the central part of the TMEM70 sequence. Two-dimensional electrophoretic analysis did not show direct interaction of TMEM70 with assembled ATP synthase but indicated the presence of dimeric form of TMEM70. No TMEM70 protein could be found in cells and isolated mitochondria from patients with ATP synthase deficiency due to TMEM70 c.317-2A>G mutation thus confirming that TMEM70 biosynthesis is prevented in these patients.

1. Introduction

Biogenesis of eukaryotic ATP synthase is a stepwise process, in which 16 different subunits assemble the structure of the enzyme consisting of the F_1 catalytic part and the membranous F_o part connected together by two stalks [1,2]. The biosynthesis of individual subunits and formation of the ATP synthase holoenzyme depend on several specific helper proteins that are partly common to, and partly unique to, higher and lower eukaryotes. Several yeast-specific factors (NCA1-3, NAM1, AEP1-3 ATP22 and ATP25) are involved in mRNA stability, translation and processing of mtDNA encoded subunits ATP6 and ATP9 [1,3–5] or their assembly (ATP10, ATP22). Additional factor ATP23 [5,6], the metalloprotease with chaperone activity is implicated in processing of ATP6 and its association with ATP9 oligomer. There exists mammalian ortholog of ATP23 which contains a HEXXH motif of the protease active site, but its function is unknown. The only two yeast factors that are found in mammals [1,7,8], having identical function are the F_1 chaperones, ATPAF1 and ATPAF2, interacting with F_1 subunits β and α. Both are absolutely essential for assembly of the functional $\alpha_3\beta_3$ heterooligomer. The FMC1, the third factor involved in F_1 assembly in yeast at high temperature [9] is again specific for yeast. Till now, only one essential ancillary factor, the TMEM70 protein, has been found in mammals being absent in yeast and fungi [10,11]. The mutations in *TMEM70* gene were found to be responsible for isolated deficiency of ATP synthase leading to a severe mitochondrial disease [10,12]. The enzyme defect was rescued by the wtTMEM70. The TMEM70 protein was identified as a putative mitochondrial protein that fulfils the criteria of MITOCARTA [13,14]. The biological roles, as well as biogenesis of this protein remain unknown. In this study we attempted to use tagged forms of TMEM70 and specific antibodies for characterization of expression, processing and localization of this factor.

2. Materials and methods

2.1. Cell cultures

Human embryonic kidney cells (HEK293, CRL-1573, ATCC) were grown in high-glucose DMEM medium (PAA) supplemented with 10% (v/v) fetal calf serum (PAA) at 37 °C in 5% CO_2 in air. Fibroblasts were grown in DMEM medium (Sigma) containing 10% fetal calf serum (Sigma), penicillin (100 U/mL) and streptomycin (100 μg/mL), at 37 °C in 5% CO_2 in air. Confluent cells were harvested by trypsinization

Abbreviations: DDM, dodecyl maltoside; F_1, catalytic part of ATP synthase; F_o, membrane embedded part of ATP synthase; PDH, pyruvate dehydrogenase

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and washed twice with PBS (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, 0.20 g/L KH_2PO_4).

2.2. Expression vectors

TMEM70 cDNA clone MHS1011-60493 was obtained from Open Biosystems. Following the sequence verification, the insert was transferred into the mammalian expression vector pEF-DEST51 using the Gateway technology (Invitrogen). Resulting plasmids TMEM70-pEF-DEST51 were propagated in *Escherichia coli*, isolated and fully sequenced before the transfection. *TMEM70-Flag* cDNA expression vector—the full-length human *TMEM70* coding sequence was amplified from the IMAGE clone 3631570 and inserted into the C-FLAG fusion mammalian expression vector pCMV-Tag4 (Stratagene). The fidelity of the construct was confirmed by sequencing. *TMEM70-GFP* cDNA expression vector [13] was kindly provided by Dr V.K. Mootha.

2.3. Transfections

Vectors were transfected into the fibroblast or HEK293 cell lines (2 μg of DNA/5×10^5 cells) using Nucleofector device and NHDF nucleofection kit (Amaxa/Lonza), following the standard protocol. For the transient expression of the TMEM70-FLAG fusion protein, cell transfection was carried out with Express-In Transfection Reagent (Open Biosystems). Transfected cell lines were cultured for 48 h on BD Falcon 4-well CultureSlides.

2.4. Isolation of mitochondria

HEK293 cells (1×10^7) were harvested by trypsinization, washed twice in PBS, re-suspended in isotonic STE buffer (250 mM sucrose, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.2% (v/v) Protease Inhibitor Cocktail (PIC, Sigma P8340)), and disrupted on ice using Dounce homogenizer. Homogenate was centrifuged for 15 min at 600*g* and 4 °C, the post-nuclear supernatant was centrifuged for 25 min at 10,000*g* and 4 °C. The resulting supernatant corresponding to the cytoplasm fraction was collected and the mitochondrial pellet was washed by centrifugation with STE buffer.

Fibroblast mitochondria were isolated by the method utilizing the hypotonic shock cell disruption [15]. To avoid proteolytic degradation, the isolation medium (250 mM sucrose, 40 mM KCl, 20 mM Tris–HCl, 2 mM EGTA, pH 7.6) was supplemented with the 0.2% PIC. The isolated mitochondria were stored at −80 °C.

Rat liver mitochondria for import studies were isolated by the method of Enriquez et al. [16].

2.5. Submitochondrial fractionation

Fractionation of mitochondria from HEK293 cells was carried out according to Satoh et al. [17] with slight modifications. Briefly, isolated mitochondria were re-suspended in STE buffer at final concentration 1 mg/mL and disrupted by repeated freezing-thawing 3 times followed by sonication on ice for 5 s at 20% amplitude and 0.5 cycle using an UP 200S Ultrasonic Processor (Hielscher, Germany). Unbroken mitochondria were removed by centrifugation at 10,000*g* for 10 min. The soluble mitochondrial proteins and membranes were separated by centrifugation of the supernatant at 100,000*g* for 35 min. The pellet was re-suspended in 100 mM sodium carbonate, pH 11.5, and incubated at 4 °C and continuous vortexing for 30 min followed by centrifugation at 100,000*g* and 4 °C for 40 min. Supernatant containing membrane associated proteins was collected and the pellet was re-suspended in STE buffer. All collected fractions were kept at −80 °C until analysis.

2.6. TMEM70 antibodies

The cDNA sequence encoding 50–260 AA of human TMEM70, corresponding to expected mature part of the protein was cloned into pMAL-c2 expression vector (New England Biolabs). The construct with the correct sequence was introduced into the *E. coli* MAX Efficiency DH5αF'IQ cells and the fusion protein MBP-TMEM70 was expressed upon IPTG induction. Fusion protein was isolated from sonicated and detergent solubilized cell lysate (20 mM Tris–HCl, 0.2 M NaCl, 1 mM EDTA, 1% Nonidet P-40) using amylose resin column (New England Biolabs). The protein was eluted from the column by 50 mM maltose and directly used for rabbit immunization (Open Biosystems).

2.7. Electrophoresis and Western blot analysis

SDS-PAGE, two-dimensional BN/SDS-PAGE and Western blot analysis were performed by standard protocols as previously [18,19] using specific primary antibodies against GFP (Santa Cruz Biotechnology), FLAG (Sigma), cytochrome *c* oxidase subunit Cox1, ATPase β subunit, PDH E1 α subunit (Mitosciences), MtHSP70 (Alexis Biochemicals), α-Tubulin (Cell Signaling), a mixture of antibodies to respiratory chain proteins (MS603 to ATPase α, Core2, NDUFA9, SDH70, Cox4; Mitosciences), or the polyclonal rabbit antibody to TMEM70. The immunoblots were detected with peroxidase-conjugated secondary antibodies and SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) using VersaDoc 4000 Imaging System (Bio-Rad), or with fluorescent secondary antibodies (Alexa Fluor 680, Molecular Probes) on an Odyssey infrared imaging system (LI-COR).

2.8. Immunocytochemistry

Fibroblast cells were grown on glass chamber slides (BD Falcon 4-well CultureSlides). After 48 h, the cells were washed with PBS, fixed and permeabilized for 10 min with methanol at −20 °C or with paraformaldehyde for 10 min at 4 °C. After blocking unspecific sites with 5% FBS, cells were incubated overnight at 4 °C with indicated antibody in 5% FBS followed by 60 min incubation at 37 °C with fluorophore-conjugated secondary antibody (1 μg/mL, Molecular Probes). The following primary antibodies were used: mouse monoclonal (MS503, Mitosciences) or polyclonal [20] to F_1 β subunit, mouse monoclonal to FLAG (Sigma), or rabbit polyclonal antibody to TMEM70.

When using MitoTracker Red (Molecular Probes), cells were incubated in 300 nM prewarmed medium solution of MitoTracker Red at 37 °C for 15 min, washed with fresh prewarmed medium, fixed and permeabilized. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Prepared slides were mounted in fluorescence mounting medium Immu-Mount (ShandonLipshaw) and analyzed by confocal microscopy (Nikon Eclipse TE2000, Leica AOBS—Acusto-Optical Beam Splitter) and/or epifluorescent microscopy (widefield epifluorescent microscope Nikon Eclipse E400).

2.9. Mitochondrial import

Protein precursor was synthesized in the presence of ^{35}S-methionine using TNT T7 Quick Coupled Transcription/Translation System (Promega) with plasmid vector or PCR product as a DNA template, according to manufacturer's recommendation. Translation product was centrifuged at 13,000*g* for 2 min and supernatant was used for import to isolated rat liver mitochondria or human HEK293 mitochondria.

The import reaction was carried out in 50 μL of a medium containing 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM KH_2PO_4, 0.05 mM EDTA, 5 mM $MgCl_2$, 10 mM Tris–HCl, 1 mg BSA/mL,

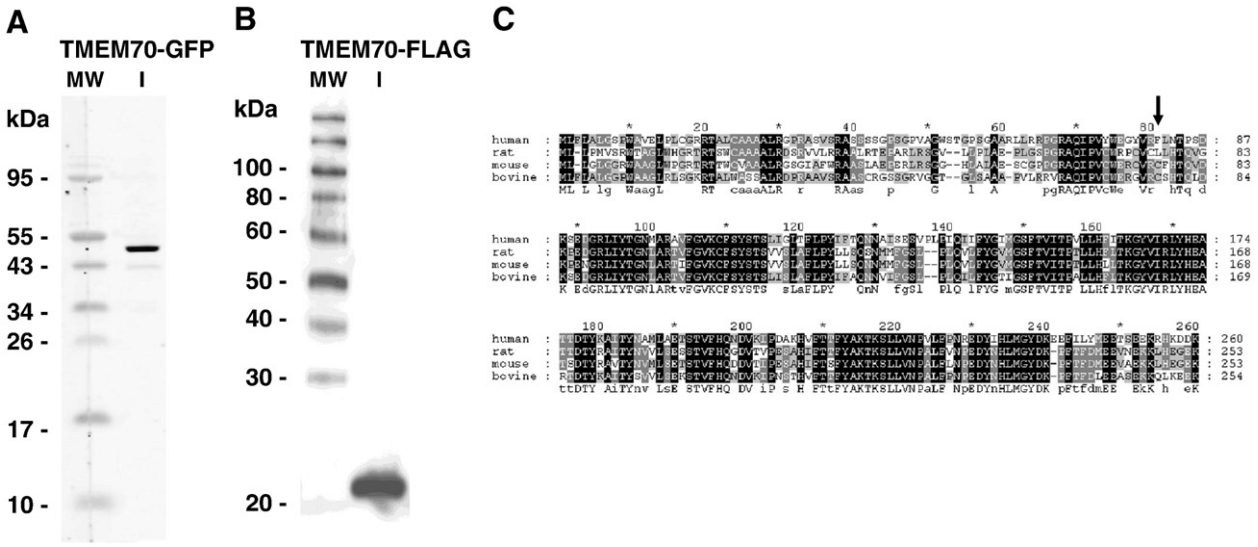

Fig. 1. The size of mature TMEM70. Western blot analysis of TMEM70-GFP (A) and TMEM70-FLAG (B) expressed in fibroblast and HEK293 cells. (C) Alignment of human (NCBI GI: 34147498), rat (157823940), mouse (15030135) and bovine (148878159) TMEM70 protein sequence; arrow indicates predicted cleavage site of the TMEM70 precursor protein.

1 mM methionine, pH 7.4, [21] and 2 mg protein/mL of freshly isolated mitochondria. Incubation with ^{35}S labeled-translation product was performed for 30 min at 30 °C, in presence or absence of 4 μM FCCP. Indicated samples were treated with 0.16 mg trypsin/mL for 20 min on ice. Then 4 μM PMSF (phenylmethanesulfonyl fluoride) was added to all samples and mitochondria were sedimented and washed twice by centrifugation at 13,000*g* for 2 min at 4 °C. Samples were analyzed by SDS-PAGE and radioactivity was detected using BAS-5000 system (Fuji).

2.10. Ethics

The project was approved by the Scientific Ethics Committees of the 1st Faculty of Medicine of Charles University in Prague and Institute of Physiology, Academy of Sciences of the Czech Republic. Patient participation in the project was made on a voluntary basis after oral and written information and consent according to the Helsinki V Declaration.

3. Results and discussion

As shown in Fig. 1A, the *TMEM70-GFP* construct is well expressed in human fibroblasts or HEK293 cells yielding a protein band of about 46 kDa. The *TMEM70* gene encodes 260 amino acids protein of expected MW of 29.0 kDa and the tagged TMEM70-GFP protein of 260 + 238 amino acids has calculated MW of 55.9 kDa. The difference between calculated and observed size of the TMEM70-GFP is ~10 kDa and corresponds well with predicted cleavable N-terminal sequence of 81 amino acids (Fig. 1C). Similar experiment using *TMEM70-FLAG* construct (Fig. 1B) revealed the size of expressed protein of approximately 22 kDa and thus both tagged forms of the TMEM70 supported the conclusion that this protein is synthesized as a precursor that is processed into a ~21 kDa mature form of 179 AA.

To obtain specific antibodies to TMEM70 protein, we have expressed mature part of TMEM70 in a form of a fusion protein with maltose binding protein (MBP-TMEM70) in *E. coli*. Resulting protein was isolated and used for immunization. As shown by SDS-PAGE/WB analysis in Fig. 2, the anti-TMEM70 antibody recognized a

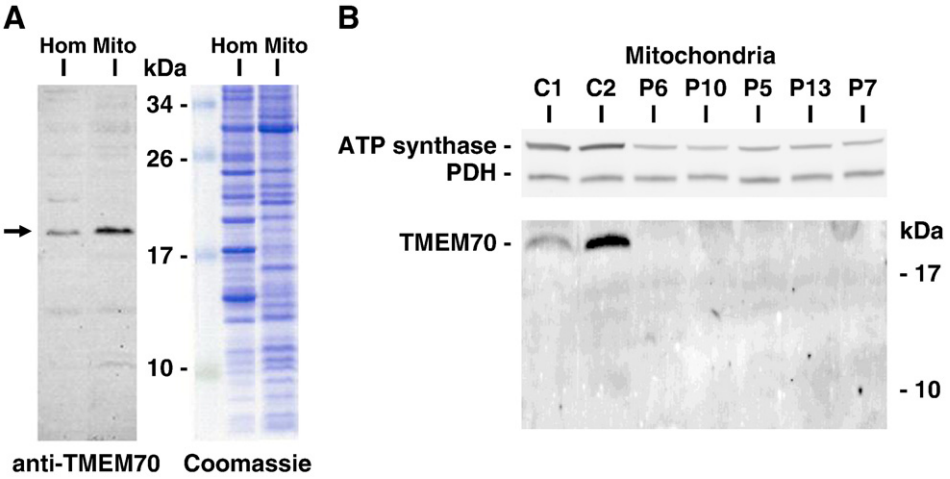

Fig. 2. Mature TMEM70 of ~21 kDa is absent in patients with ATP synthase deficiency. (A) Western blot detection of TMEM70 by polyclonal antibody in human heart homogenate and mitochondria. (B) Western blot detection of ATP synthase (β subunit), PDH (E1 α subunit) and TMEM70 in fibroblast mitochondria from control (C1, C2) and indicated patients (P) with *TMEM70* c.317-2A>G mutation.

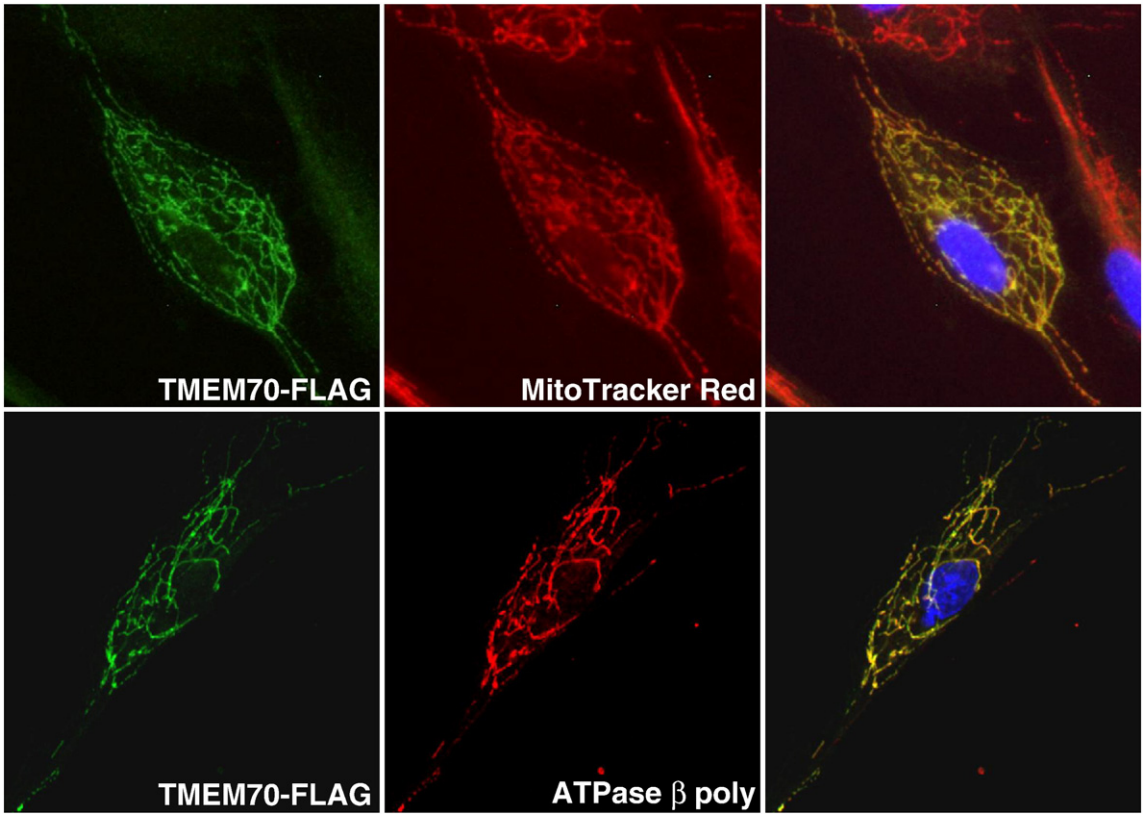

Fig. 3. Subcellular localization of TMEM70-FLAG with respect to MitoTracker Red or ATP synthase in human fibroblasts. Last column represents an overlay of the first two columns and shows cell nuclei stained with DAPI (blue).

20 kDa band in tissue homogenate of control human heart that was concentrated in isolated mitochondria (10,000*g*). The immunoreactive band was absent in fibroblast mitochondria (Fig. 2B) of the patients with ATP synthase deficiency due to the homozygous *TMEM70* mutation c.317-2A>G. This mutation at the second intron of TMEM70 gene has been shown to results in aberrant splicing and loss of *TMEM70* mRNA [10]. WB with anti-TMEM70 antibody thus confirmed previous conclusion that normal TMEM70 protein is absent in patient mitochondria. There was no immunoreactive band at lower molecular weight region indicating that no aberrant TMEM70 protein is produced in patient cells.

The presence of TMEM70 protein was also verified by mass spectrometry. LC-MS/MS analysis performed on mitochondria, whole tissue homogenates or 100,000*g* microsomal fraction from human heart and mouse heart or liver did not detect the TMEM70 protein. However, when a targeted approach was used (Supplementary Fig. S1), based on the knowledge of the retention time, precise mass and fragmentation spectrum of the human TMEM70 protein (MS/MS analysis of MBP-humanTMEM70 fusion protein), TMEM70 HVFTTFYAK tryptic peptide that is not present in any other human protein was found in the approximately 18–23 kDa region sample of isolated human heart mitochondria. MS analysis thus confirmed mitochondrial location of TMEM70 and further indicated that the cellular content of the TMEM70 is very low.

This conclusion is also supported by existing expression profile data (http://biogps.gnf.org/), which show that the level of *TMEM70* transcripts is extremely low in human cells and tissues (Supplementary Fig. S2), being several orders of magnitude lower than the level of mRNAs for structural subunits of ATP synthase (e.g. *ATP5B* mRNA for F_1 β subunit). The *TMEM70* mRNA levels are thus similarly low as the levels of transcripts of *ATPAF1* and *ATPAF2* assembly factors [1], the content of which is very small in mammalian tissues (Supplementary Fig. S2 and [22]). Furthermore, *TMEM70* transcripts show very small variation among various tissues, similarly as *ATPAF2*, characteristic for housekeeping genes. All these data thus support the view that the TMEM70 protein, ancillary factor of ATP synthase biogenesis is a low abundant mitochondrial protein, not exerting tissue-specificity.

Cellular localization of TMEM70 protein was further analyzed at a morphological level in cultured fibroblasts. For experiments the cells transfected with tagged *TMEM70* or control cells expressing the *wtTMEM70* were used. As shown in Fig. 3, in cells transfected with

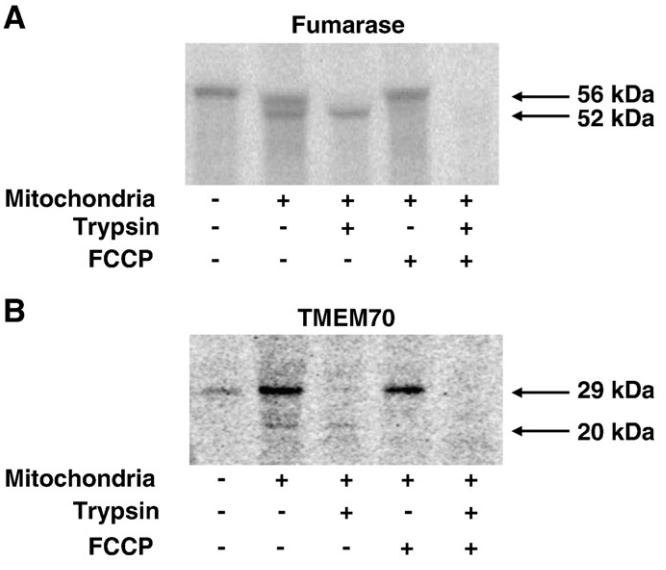

Fig. 4. Import of TMEM70 to mitochondria. *In vitro* translated human fumarase (A) and TMEM70 (B) were processed and imported to isolated rat liver mitochondria. Upon the import, the mature forms of both proteins resisted to trypsin and their import was prevented by uncoupler of oxidative phosphorylation FCCP.

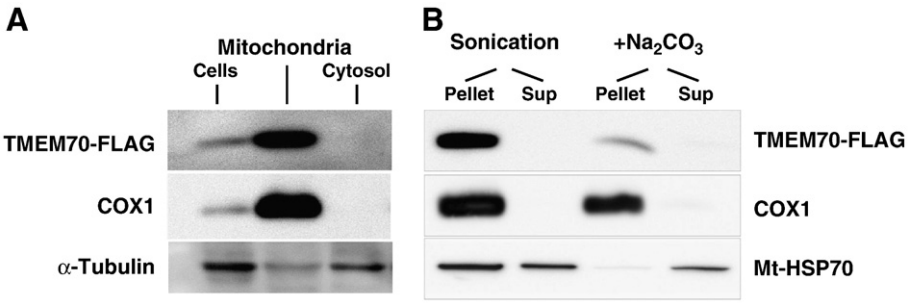

Fig. 5. TMEM70 is a membrane bound mitochondrial protein. HEK293 cells were transfected with *TMEM70-FLAG* and (A) cell homogenate, isolated mitochondria and cytosolic fractions were analyzed. (B) Isolated mitochondria were sonicated and extracted with Na_2CO_3 and 100,000*g* pellet and supernatant were prepared. Western blot was performed with indicated antibodies.

TMEM70-FLAG the tagged TMEM70 signal detected by anti-FLAG antibody colocalized with the signal of MitoTracker Red and with the signal of mitochondrial ATP synthase, detected with antibody to β subunit of the F_1 catalytic part, in accordance with previously demonstrated mitochondrial localization of TMEM70-GFP [13]. Antibody to TMEM70 protein also confirmed mitochondrial localization in control cells, but it was less specific in immunocytochemical experiments (not shown).

To assess further the basic properties of TMEM70 import to mitochondria, we have synthesized radioactive TMEM70 *in vitro* using coupled transcription-translation system and followed its import to isolated mitochondria. For comparison, we analyzed import of matrix located fumarase, as an example of mitochondrial protein that is synthesized with cleavable N-terminal sequence. As shown in Fig. 4A, the import analyses with isolated liver mitochondria showed a 56 kDa precursor and a 52 kDa mature form of fumarase, with expected sensitivity to trypsin and/or uncoupler. Analysis of TMEM70 protein revealed ~29 kDa band produced by *in vitro* translation and additional band of ~20 kDa present in the mitochondrial pellet. The 29 kDa band was sensitive to protease indicating its extramitochondrial localization typical for a precursor form. The 20 kDa band was resistant to protease but it disappeared in the presence of uncoupler, thus confirming the intramitochondrial localization of the TMEM70 mature form. Interestingly, there was only small amount of the mature 20 kDa protein found relative to the amount of precursor added. These data indicate that upon cleavage of the N-terminal part, the mature TMEM70 is rather labile at the conditions of *in vitro* import assay, or that additional cellular components are required for its import *in vivo*. However, similar pattern was observed when using mitochondria of human origin isolated from HEK293 cells or in co-translational import assay.

To characterize further the mitochondrial localization of TMEM70, we have investigated HEK293 cells expressing the *TMEM70-FLAG* construct, which is only slightly larger than the *wtTMEM70*. When we have isolated mitochondria from the cells expressing the TMEM70-FLAG protein, we found that the TMEM70-FLAG was fully recovered in mitochondria while it was absent in the cytosolic fraction (Fig. 5A). Then we fractionated the isolated mitochondria by sonication and treated the mitochondrial membranes with Na_2CO_3. Analysis of 100,000*g* pellet and supernatant by SDS-PAGE and WB (Fig. 5B) fully recovered the TMEM70-FLAG in the sediment, similarly as cytochrome *c* oxidase (COX), indicating its localization in the mitochondrial membrane. Efficiency of the treatments was verified by antibody to matrix located HSP70 that was quantitatively recovered in the soluble fractions.

Finally, to search for native conformation of TMEM70, we have extracted fibroblast mitochondria with mild detergent dodecyl maltoside (DDM) and analyzed the solubilized proteins by two-dimensional BN-PAGE/SDS-PAGE and WB. As demonstrated in Fig. 6, TMEM70 was found as two spots of identical mobility in the second

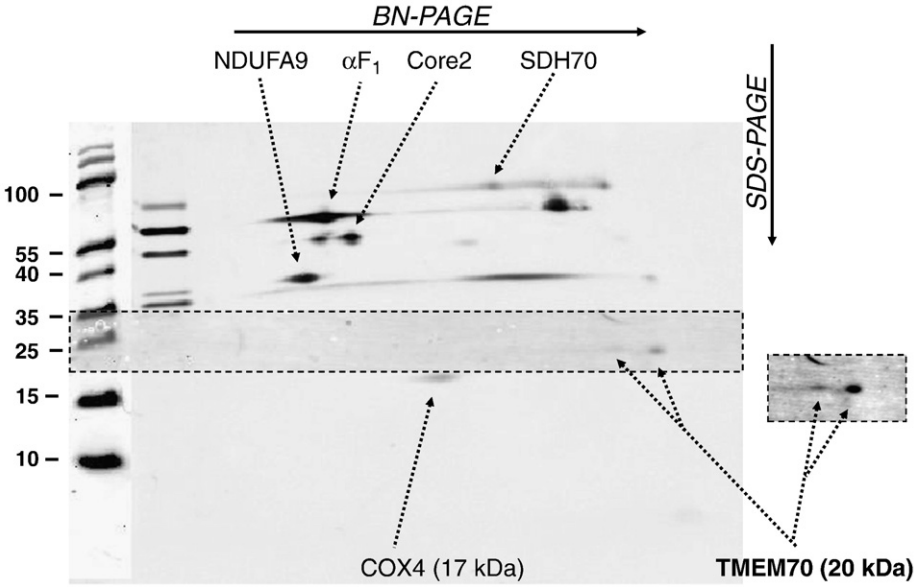

Fig. 6. DDM-solubilized mitochondrial proteins reveal a dimeric form of TMEM70. Fibroblast mitochondria were solubilized with DDM (1 g/g protein) and 30,000*g* supernatant was analyzed by two-dimensional electrophoresis (BN/SDS-PAGE) and WB using antibodies to indicated proteins. Dashed line frames the region probed with anti-TMEM70 antibody (higher intensity signal shown on the right).

dimension, corresponding to 21 kDa. Their mobility in the first dimension BN-PAGE gel was approximately 20 and 40 kDa. Parallel detection of ATP synthase and respiratory chain complexes did not reveal any association of TMEM70 with the assembled ATP synthase complex or free α or β subunits and indicated that TMEM70 exists in monomeric and dimeric forms when solubilized with detergent DDM.

Taken together, our experiments convincingly demonstrated that TMEM70 is a membrane bound 21 kDa mitochondrial protein that is synthesized as a 29 kDa precursor. TMEM70 is firmly associated with inner mitochondrial membrane and it does not interact directly with the ATP synthase complex. Very low cellular content of this protein, analogous to low content of ATPAF1 and ATPAF2 chaperones [23] supports the view of a regulatory — catalytic role of TMEM70 in ATP synthase biogenesis. It also indicates that putative interacting partner of TMEM70 might be similarly low-abundant protein(s), as are for example F_1 assembly intermediates [24]. A larger form of TMEM70 on two-dimensional gels appears to be a dimer according to calculated molecular weight, but we cannot exclude that it represents TMEM70 interacting with some other protein, although it cannot be the large ATP synthase subunits. Absence of TMEM70 in patient cells containing small amounts of functional ATP synthase complex [10,11] indicates that TMEM70 is not absolutely essential for ATP synthase biogenesis. Further studies are needed to elucidate the biological role of this factor. A unique specificity of TMEM70 to higher eukaryotes prevents the use of yeast cells, but the studies utilizing overexpression of TMEM70, various tagged forms as well as crosslinking and preparation of antibodies allowing for specific immunoprecipitation may represent a perspective strategy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbabio.2010.10.005.

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